

**EXHIBIT C**

## Platelet-derived Microparticle Formation Involves Glycoprotein IIb-IIIa

INHIBITION BY RGDS AND A GLANZMANN'S THROMBASTHENIA DEFECT\*

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While the physiologic role of platelet microparticles may include a stable, physical dispersion of concentrated surface procoagulant activity the mechanism(s) of platelet vesiculation remains unknown. We demonstrate using flow cytometric methods a central role for the  $\beta_3$  integrin glycoprotein (GP) IIb-IIIa complex and its ligand tetrapeptide Arg-Gly-Asp-Ser (RGDS) binding site in platelet vesiculation. Time- and calcium-dependent vesiculation of platelets in response to ADP, collagen, thrombin, phorbol myristate acetate, and the thrombin peptide SFLLRN were dramatically inhibited, in a concentration-dependent manner, by monoclonal antibodies to GPIIb-IIIa (A2A9, 7E3, PAC1) and RGDS. Complete inhibition with A2A9 and RGDS occurred at 7.5  $\mu\text{g}/\text{ml}$  and 75  $\mu\text{M}$ , respectively, while control antibodies and a mock peptide had no effect. Platelet vesiculation requires intact GPIIb-IIIa and is fully supported by the intracellular pool of GPIIb-IIIa alone since de-complexing of this heterodimer by calcium chelation completely abolished microparticle formation in response to collagen (no  $\alpha$ -granule release) but not to thrombin or SFLLRN. A central role for GPIIb-IIIa is supported by the near total inability of Glanzmann's thrombasthenic (type I) platelets to vesiculate in response to thrombin, ADP, collagen, and phorbol 12-myristate 13-acetate. This extends the biologic roles of GPIIb-IIIa to include platelet vesiculation and suggests that one or all of its binding ligands play a role.

Platelet activation elicits a variety of physiologic cellular responses including shape change, intracellular ion fluxes, induction of coupling responses, biochemical membrane alterations, induction of membrane procoagulant activity, release of granule contents, and initiation of aggregation (1). The release from the cell surface of small membrane vesicles or microparticles should be added to the list (1). Platelet vesicles, initially observed in electron micrographs (2–5), were characterized as procoagulant in 1985 (6). With the technological advances of fluorescence-gated flow cytometry investigators have shown

that agonists such as complement, thrombin, collagen, and the calcium ionophore A23187 induce platelets to vesiculate (1).

Microparticles have been observed *in vivo* in clinical conditions associated with platelet activation including idiopathic thrombocytopenia purpura (7), transient ischemic attacks (8) and during cardiopulmonary bypass (9). The biologic function of microparticles remains speculative, but the tenase and prothrombinase activity including the factor Va, high affinity factor IXa, and factor VIII activity is concentrated on these vesicles (1, 10–14). In addition microparticles have anticoagulant activity since they inactivate prothrombinase (15) by activated protein C. These observations suggest microparticles may play a role in modulating hemostasis and thrombosis (16).

The mechanism(s) by which platelet membranes vesiculate remains unresolved. We report that intact activated GPIIb-IIIa<sup>1</sup> heterodimer complex, the  $\beta_3$  integrin adhesive ligand receptor for fibrinogen, von Willebrand factor, fibronectin, and vitronectin, with a surface density of 40,000 complexes per platelet, plays a central role in platelet microparticle formation (17). Microparticle generation was fully inhibited by both monoclonal antibodies to GPIIb-IIIa and the common tetrapeptide Arg-Gly-Asp-Ser (RGDS) sequence of the GPIIb-IIIa ligands. These observations were supported by the inability of type I Glanzmann's thrombasthenic platelets, with less than 0.5% GPIIb/IIIa, to vesiculate. We hypothesize that one or all of the GPIIb-IIIa adhesive ligands will be involved in platelet vesiculation.

### EXPERIMENTAL PROCEDURES

**Materials**—Bovine thrombin was from Parke-Davis, Scarborough, Ontario; fibronectin inhibitor RGDS, H-Gly-Arg-Ala-Asp-Ser-Pro-OH (GRANSP mock peptide), and D-phenylalanyl-L-prolyl-arginyl chloromethyl ketone (PPACK) from Calbiochem; thrombin peptide, Ser-Phe-Leu-Arg-Asp (SFLLRN), was synthesized by the University of Toronto Peptide Synthesis Centre; heparin (porcine) from Diosynth Inc., Chicago, IL. All other chemicals were of analytical or reagent grade.

**Antibodies**—Monoclonal antibodies (mAb) to: GPIb (AP1 (18)), provided by Dr. T. Kunicki, Blood Center of Southeastern Wisconsin; GPIIb-IIIa complex (7E3 (19)), provided by Dr. B. Coller, Stony Brook University, NY; GPIIb-IIIa complex (A2A9 (20)), provided by Dr. J. Bennett, University of Pennsylvania and GPIIb-IIIa (PAC1 (20)), provided by Dr. S. Shattil, University of Pennsylvania have been previously described. The mAb to 8A3 that recognizes a glycoprotein of  $M_r$  180,000 on activated but not resting platelets (22) and to P-selectin (KC<sub>4.1</sub> (21, 22)) were provided by E. L. Y. An irrelevant monoclonal antibody of the same isotype was used as a control. For flow cytometry studies mAb were conjugated with either fluorescein isothiocyanate (FITC-celite, Calbiochem) (F/P molar ratios 5–10) or with R-phycoerythrin (Molecular Probes) using the methodology of Molecular Probes (23).

**Platelet Preparation**—Whole blood from normal volunteers and one Glanzmann's thrombasthenic (GT) was anticoagulated with the selective thrombin inhibitor 60  $\mu\text{M}$  PPACK or 10 units/ml heparin. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood for 5 min at 160  $\times g$ . Gel-filtered platelets (GFP) were prepared following standard methods (21) by applying PRP to a column of Sepharose 2B (Pharmacia LKB Biotechnology Inc.) pre-equilibrated with a HEPES buffer (HTB: 137 mM NaCl, 2.7 mM KCl, 5.0 mM  $\text{MgCl}_2$ , 16 mM  $\text{NaHCO}_3$ , 1 g/liter glucose, 2 g/liter albumin, 35 mM HEPES, pH 7.4). The GFP (2.5  $\times 10^6/\text{mL}$ ) was used immediately following the addition of 2.5 mM  $\text{Ca}^{2+}$ .

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<sup>1</sup> The abbreviations used are: GP, glycoprotein; PMA, phorbol 12-myristate 13-acetate; PPACK, D-phenylalanyl-L-prolyl-arginyl chloromethyl ketone; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; GT, Glanzmann's thrombasthenic; PRP, platelet-rich plasma; GFP, gel-filtered platelets; MP, microparticle(s); Fl. U., fluorescence units.

To irreversibly de-complex surface GPIIb-IIIa (24), PRP was incubated with 10 mM EDTA at 37 °C for 30 min, and the platelets either washed by centrifugation (300 × g for 10 min) and resuspended in fresh plasma or gel-filtered. Loss of surface GPIIb-IIIa complex was monitored by flow cytometry with FITC-A2A9 and judged to be greater than 99% complete by 10 min. Platelets remained de-complexed thereafter in the re-calcified milieu. The type I Glanzmann's thrombasthenic platelets from patient U.W. had less than 0.5% immunoreactive GPIIb-IIIa analyzed by both immunoprecipitation of radiolabeled membranes and flow cytometry. U.W. platelets failed to aggregate in the presence of ADP, collagen, epinephrine, and arachidonic acid but expressed normal amounts of P-selectin and 8A3 upon activation.

**Generation of Platelet Microparticles**—To generate microparticles (MPs), 100 µl of platelet medium, in the presence or absence of agonist 0.5 µM PMA, 20 µM ADP, 200 µg/ml collagen, 0.25 units/ml thrombin, or 100 µM SFLLRN, were mixed and then incubated at 37 °C unless otherwise stated. For inhibition studies, mAb and peptides, at different concentrations, were added 5 min prior to the addition of the agonist. After 15 min aliquots were diluted in HTB containing saturating concentrations of fluorochrome-labeled mAb, incubated for 20 min, diluted and fixed in 1% fresh paraformaldehyde, and analyzed by flow cytometry. For dual label flow cytometric studies R-phycoerythrin and FITC-labeled antibodies were added simultaneously.

**Flow Cytometry**—Samples were analyzed on a Becton Dickinson FACScan flow cytometer (Mountain View, CA) formatted for two-color analysis. The light scatter and fluorescence channels were set at logarithmic gain. Platelet-specific events, including microparticles, were identified by gating on GPIb (FITC-AP1) or GPIIb-IIIa (FITC-A2A9) positive events. Single intact platelets were distinguished from microparticles by forward scatter size analysis. 5,000 positive platelet events were analyzed, mean fluorescence units (Fl. U.) quantitated, and microparticles reported as a percent of total platelet events. MPs were also quantitated by forward scatter size analysis alone for comparison to immunocytochemical data. Studies were triplicated at a minimum.

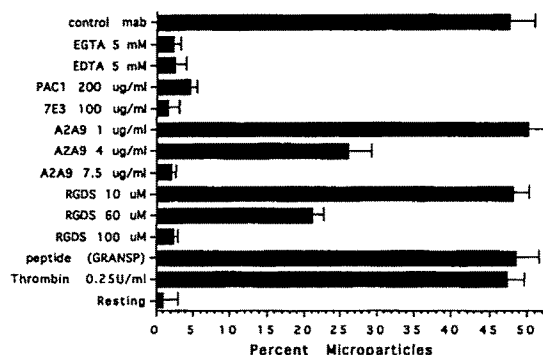
## RESULTS

**Effect of GPIIb-IIIa Antibodies and Peptides on Microparticle Formation**—Platelet vesiculation was a calcium-dependent activation event since 5 mM EDTA and 5 mM EGTA completely inhibited thrombin-induced vesiculation in GFP to the level of resting samples (Fig. 1). As described by others we found initial mixing to be an important variable in maximizing MP formation (15). MP formation in thrombin-stimulated GFP (48 ± 5% of total platelet events) was inhibited by mAbs to GPIIb-IIIa complex (7E3, A2A9), activated GPIIb-IIIa (PAC1) (20), and the GPIIb-IIIa ligand binding site tetrapeptide RGDS in a concentration-dependent manner. Inhibition with A2A9 and RGDS was half-maximal at 4 µg/ml and 50 µM and maximal at 7.5 µg/ml and 75 µM, respectively. Inhibition was near complete

being less than 2% greater than the resting samples and to the same extent as calcium ion chelation (Fig. 1). The A2A9 and RGDS concentrations required for MP inhibition are in the range reported for their inhibition of fibrinogen binding to activated GPIIb-IIIa (25, 26). The control mock peptide (GRANSP) and control mAb had no effect. We saw a similar effect on MP formation with RGDS, A2A9, 7E3, and PAC1 with the agonists ADP, collagen, PMA, and thrombin peptide SFLLRN in the more physiologic milieu of PRP (data not shown). These data suggest a role for both activated GPIIb-IIIa and its adhesive ligands in MP formation since RGDS and antibodies are known to inhibit ligand binding to GPIIb-IIIa (17, 25, 26).

**Role of Intact GPIIb-IIIa in Microparticle Formation**—Since our data suggested a role for the combination of GPIIb-IIIa and its adhesive ligands in MP formation we sought to separate the activated platelet surface GPIIb-IIIa complexes from potential binding ligands available in plasma or by α-granule secretion. We compared MP response, in the presence and absence of de-complexed surface GPIIb-IIIa with plasma or buffer and two agonists, collagen, which induced no α-granule release, and thrombin peptide SFLLRN, which results in complete α-granule release and surface expression of an internal pool of GPIIb-IIIa receptors. We determined whether GPIIb or GPIIIa in their uncomplexed form could support MP formation. Resting samples had few MPs (3%) and a very low level of α-granule release based on P-selectin expression while de-complexation of GPIIb-IIIa receptors (24) was greater than 98% complete (2.6 versus control 241 Fl. U.) and remained irreversible throughout our experiments (Table I). Collagen stimulation of control and de-complexed platelets resulted in less than 1% P-selectin (α-granule secretion) and GPIIb-IIIa surface up-regulation. Collagen-induced vesiculation (32 ± 9%) occurred only in the combination of intact surface GPIIb-IIIa and plasma but not buffer. In contrast, SFLLRN stimulation of platelets resulted in complete vesiculation in the presence or absence of plasma regardless of de-complexation. SFLLRN stimulation of de-complexed platelets resulted in P-selectin expression (99.9 versus control 97.6 Fl. U.) and up-regulation of GPIIb-IIIa surface expression (97.2 versus resting 2.6 Fl. U.), indicating near complete secretion of α-granules and mobilization of the intracellular pool of GPIIb-IIIa (27). MP generation of de-complexed or control platelets with collagen or SFLLRN was fully inhibited with RGDS or A2A9. These results indicate that the combination of intact activated GPIIb-IIIa complex and a GPIIb-IIIa binding component supplied either by the plasma or α-granule is required for vesiculation. Furthermore, the intracellular pool of GPIIb-IIIa complex alone, which represented 29% of total GPIIb-IIIa pool, was sufficient to support this biologic response. The minimal surface GPIIb-IIIa that will support MP formation is under study.

**Microparticle Formation in Glanzmann's Thrombasthenia**—We noted a dramatic difference in MP formation between type I GT platelets with less than 0.5% GPIIb-IIIa and normal platelets. Thrombin-induced MP formation in Glanzmann's GFP in 30 min was dramatically reduced at 6.13% versus control of 53.4% while the unstimulated control was 3.02% (Fig. 2). MP generation in PRP anticoagulated with heparin was compared with the agonists: ADP, collagen, and PMA (Fig. 3). Vesiculation in normal PRP with ADP was weaker than with PMA or collagen. MP formation in Glanzmann's PRP was dramatically reduced with all agonists for times up to 60 min, the maximal being less than 5% above that spontaneously generated in resting samples (Fig. 3). MP generation in control samples was fully inhibited by RGDS, A2A9, and EDTA, while in GT samples these conditions had little or no effect (data not



**FIG. 1. The effects of different antibodies and peptides on thrombin-induced microparticle formation.** GFP was incubated with thrombin in the presence of: EDTA, EGTA, GPIIb-IIIa antibodies (A2A9, 7E3, PAC1), RGDS, control antibodies, and peptide. At 15 min, aliquots were incubated with saturating concentrations of FITC-AP1 (anti-GPIb) for 20 min, fixed, and 5,000 FITC-positive events analyzed by flow cytometry. MPs, defined as positive FITC-events smaller (based on forward scatter) than intact single platelets, are expressed as a percent of total GPIb platelet events. The data are a mean of five experiments ± S.E.

TABLE I

Platelet microparticle generation versus GPIIb-IIIa and P-selectin content on GPIIb-IIIa de-complexed platelets

Platelets (PRP) were GPIIb-IIIa-de-complexed (37 °C, 10 mM EDTA for 30 min), washed, and reconstituted in HTB pH 7.4, 2.5 mM Ca<sup>2+</sup> or PPACK fresh platelet-poor plasma. Platelets were incubated with saturating concentrations of FITC-A2A9 (GPIIb-IIIa) or FITC-AP1 (GPIIb) and PE-KC4.1 (P-selectin) in the presence of collagen, the thrombin peptide SFLLRN, or buffer, then analyzed by two-color flow cytometry. P-selectin and GPIIb-IIIa expression are expressed as arbitrary fluorescence units per platelet. Microparticles, identified by GPIIb binding and size characteristics, are expressed as a percent of total platelet events. The data are the mean of five experiments  $\pm$  S.E.

	Percent microparticles				Platelet fluorescence arbitrary units			
	Control platelets		GPIIb-IIIa de-complexed platelets		Control platelets		GPIIb-IIIa de-complexed platelets	
	Plasma	Buffer	Plasma	Buffer	P-selectin	GPIIb-IIIa	P-selectin	GPIIb-IIIa
Resting	3.1 $\pm$ 0.7	2.8 $\pm$ 0.8	3.3 $\pm$ 0.9	2.9 $\pm$ 0.6	5.3 $\pm$ 1.7	241 $\pm$ 22	5.7 $\pm$ 1.5	2.6 $\pm$ 2.5
Collagen	32 $\pm$ 9	3.2 $\pm$ 0.7	3.3 $\pm$ 0.7	3.4 $\pm$ 0.5	6.4 $\pm$ 1.7	232 $\pm$ 23	6.7 $\pm$ 1.8	3.8 $\pm$ 2.7
SFLLRN	53 $\pm$ 7	48 $\pm$ 9	55 $\pm$ 7	51 $\pm$ 6	97.6 $\pm$ 8.7	330 $\pm$ 25	99.9 $\pm$ 8.3	97.2 $\pm$ 10.1

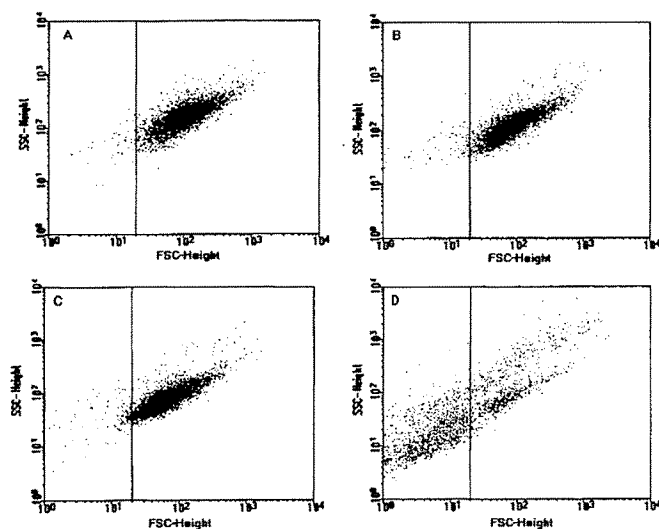


FIG. 2. Representative dot plots of normal (B, D) and GT (A, C) platelet events in resting (A, B) and activated (C, D) samples. GFP in the presence and absence of thrombin was incubated with saturating concentrations of FITC-AP1 (anti-GPIIb) fixed, and 5,000 FITC positive events analyzed by flow cytometry. Microparticles (to the left of vertical line) were defined as GPIIb positive events smaller than intact single platelets (to the right of vertical line).

shown). Thrombin stimulated GT platelets compared to controls, although defective in MP formation and the expression of complexed GPIIb/IIIa (A2A9: 1.7  $\pm$  0.9 versus 219  $\pm$  22 Fl. U.) and activated GPIIb/IIIa (PAC1: 3.5  $\pm$  1.3 versus 29.5  $\pm$  3.1 Fl. U.) per platelet, exhibited normal surface content of P-selectin (KC4.1: 29.3  $\pm$  2.8 versus 32.4  $\pm$  3.5 Fl. U.) and the platelet activation glycoprotein GP170 (8A3: 6.7  $\pm$  1.3 versus 7.1  $\pm$  0.9 Fl. U.). The failure of GT platelets, which lacked GPIIb-IIIa, to vesiculate is in agreement with our peptide and GPIIb-IIIa antibody inhibition studies and underscores a central role for this  $\beta_3$  integrin in platelet MP formation.

## DISCUSSION

Platelet microparticles (platelet dust or vesicles) are generated by unknown mechanisms from activated platelets *in vitro* and in clinical states of platelet activation (1, 6–9). We have shown that regardless of platelet medium or agonist (ADP, collagen, PMA, thrombin, and the thrombin peptide SFLLRN), the calcium-dependent generation of platelet vesicles was fully inhibited by mAbs to the  $\beta_3$  integrin GPIIb-IIIa heterodimer complex and the GPIIb-IIIa adhesion ligand tetrapeptide RGDS. The platelet surface GPIIb-IIIa heterodimer, upon activation, undergoes calcium-dependent conformational changes to express binding sites defined by the antibody PAC1 (20) and the RGDS peptide (28) for the adhesive ligands fibrinogen, von Willebrand factor, fibronectin, and vitronectin (29). We report

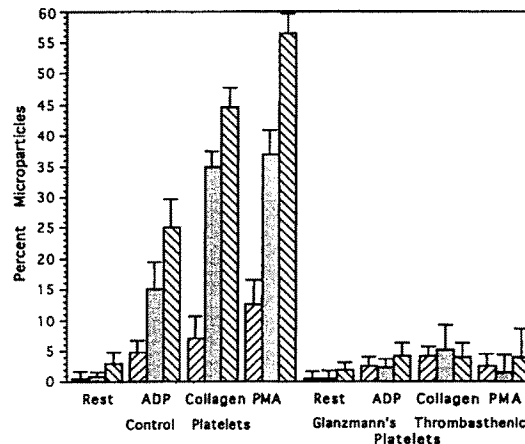


FIG. 3. A comparison of resting and agonist-induced (20  $\mu$ M ADP, 200  $\mu$ g/ml collagen, 0.5  $\mu$ M PMA) microparticle formation over 1 h in PRP between normal and GT platelets. Aliquots at time intervals (15, 30, and 60 min) were incubated with saturating concentrations of FITC-AP1 (anti-GPIIb), fixed, and 5,000 FITC-AP1 positive events analyzed by flow cytometry. Microparticles, defined as FITC positive events smaller (based on forward scatter) than single platelets, are expressed as a percent of total GPIIb platelet events. Controls represent a mean of three separate experiments  $\pm$  S.E. GT data are triplicated samples  $\pm$  S.E. for the Glanzmann's platelets.

that the antibody PAC1, whose binding is blocked by RGDS and recognizes the activated form of GPIIb-IIIa, in addition to antibodies against the resting or activated complex (A2A9, 7E3) inhibits MP formation.

A role for a GPIIb-IIIa adhesive ligand (fibrinogen, fibronectin, von Willebrand factor, and vitronectin) is suggested by our observation that the antibody A2A9 and RGDS peptide concentrations required to inhibit vesiculation were in the range that has been reported to inhibit ligand binding to GPIIb-IIIa (25, 26). Further, we show the combination of both available surface GPIIb-IIIa and a source of ligand either from plasma or secreted  $\alpha$ -granules was required for MP formation. This may explain why we were able to generate vesicles with ADP and collagen in plasma but not buffer. Collagen has been shown to vesiculate platelets in buffer but was accompanied by significant mechanically induced  $\alpha$ -granule release (6).

Intact GPIIb-IIIa heterodimer complex is required for MP formation since de-complexation of surface GPIIb-IIIa receptors led to an inability of platelets, in buffer or plasma, to vesiculate in response to stimulation with collagen, an agonist that induces no  $\alpha$ -granule release. In contrast, stimulation with thrombin or the thrombin peptide SFLLRN, which resulted in complete  $\alpha$ -granule release and the surface expression of an internal pool of intact GPIIb-IIIa receptors, led to a degree of vesiculation comparable with samples with fully intact surface GPIIb-IIIa. Thus the internal pool of complexed GPIIb-IIIa was sufficient to support full vesiculation.

A central role for GPIIb-IIIa in platelet vesiculation is supported by our observation that type I Glanzmann's thrombasthenic platelets, with less than 0.5% GPIIb-IIIa, were dramatically impaired in microparticle generation to all agonists. GT is a platelet bleeding disorder due to a rare autosomal recessive lack of GPIIb-IIIa receptors (30). Since MPs express hemostatic surface properties, the failure of Glanzmann's platelets to vesiculate may play a role in their bleeding diatheses. These observations would expand the defects of Glanzmann's thrombasthenia to include microparticle generation. While the minimal surface density of GPIIb-IIIa required to support MP formation is unknown, we hypothesize that type II Glanzmann's, with 5–25% GPIIb-IIIa, will vesiculate to some degree since the intracellular pool of GPIIb-IIIa alone supports vesiculation.

Scott's syndrome has been reported to have impaired microparticle formation (1). In this rare bleeding disorder (31) platelets have a 75% reduction in catalytic surface for prothrombinase and tenase due to a defect in phosphatidylserine surface exposure (32). Microparticles in Scott's syndrome, although decreased in number, were generated (1). In contrast, we have shown that GT platelets completely failed to generate microparticles (Figs. 2 and 3). Prothrombinase activity in Glanzmann's platelets has been reported as normal although earlier literature is controversial (33). While Scott's syndrome has normal GPIIb-IIIa expression other abnormalities including defective proteolysis of cytoskeletal proteins have been described (34). An additional defect in Scott's syndrome involving intracellular coupling responses of GPIIb-IIIa, ligand binding, or cytoskeletal formation may explain the decreased vesiculation seen.

Consistent with the concept that vesiculation is a platelet activation event, the addition of platelet activator inhibitors, prostaglandin E-1, theophylline, and aprotinin, reduced by 40% the number of microparticles appearing during stored platelet concentrates (35). Ligand binding induces biophysical and biochemical changes in the GPIIb-IIIa receptor (36) that lead to platelet functional responses (37). We hypothesize that a ligand coupling response, via GPIIb-IIIa, possibly leading to a cellular mechanism for exposure of acidic phospholipids on membrane surfaces (12) and intracellular signaling with cytoskeletal formation may be required for vesiculation. Since fibrinogen is the dominant GPIIb-IIIa ligand found both in plasma and secreted from the  $\alpha$ -granule it is a prime candidate as the ligand in microparticle formation. Studies to characterize the role of GPIIb-IIIa adhesive ligands in MP formation are ongoing. Following RGDS peptide and fibrinogen binding to stimulated platelets, the GPIIb-IIIa heterodimers become clustered (38). While clustering of GPIIb-IIIa may be involved, the inhibition of vesiculation following RGDS binding in our experiments suggests additional GPIIb-IIIa activation events are required. Although proteolytic degradation of cytoskeletal proteins does not appear to be involved (39), indications are that kinase reactions facilitate the calcium-dependent step(s) in vesiculation (11). The differences in the degree of vesiculation with ADP, collagen, PMA, and thrombin do not appear to correlate with the known differences in each agonist's ability to activate GPIIb-IIIa and induce granule secretion, suggesting other agonist-specific mechanisms may be involved.

Platelet vesiculation is a platelet activation event with a presumptive role in hemostasis involving physical dispersion of concentrated procoagulant and anticoagulant surface activity. Our data indicate that the mechanism(s) of microparticle formation requires intact GPIIb-IIIa complex and the occupancy of GPIIa/IIIb receptor with an adhesion ligand. We hypothesize

that fibrinogen will be that ligand. Platelets lacking GPIIb-IIIa, from a Glanzmann's thrombasthenic individual with a significant bleeding diathesis, were fully impaired in their ability to vesiculate. The Glanzmann observations support the requirement of GPIIb-IIIa in microparticle formation and suggest a significant role for platelet microparticles in hemostasis. These studies expand the roles of the platelet  $\beta_3$  integrin GPIIb-IIIa complex to include microparticle formation.

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